# The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae, glycinea,* and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean

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The *Pseudomonas syringae* pathovars are composed of host-specific plant pathogens that characteristically elicit the defense-associated hypersensitive response (HR) in nonhost plants. *P. s.* pv. *syringae* 61 secretes an HR elicitor, harpin$_{Pss}$ (HrpZ$_{Pss}$), in a *hrp*-dependent manner. An internal fragment of the *P. s.* pv. *syringae* 61 *hrpZ* gene was used to clone the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 (bacterial blight of soybean) and *P. s.* pv. *tomato* DC3000 (bacterial speck of tomato). DNA sequence analysis revealed that *hrpZ* is the second ORF in a polycistronic operon. The amino acid sequence identities of HrpZ$_{Pss}$/HrpZ$_{Psg}$ and HrpZ$_{Pss}$/HrpZ$_{Pst}$ were 79 and 63%, respectively. Although none of the HrpZ proteins showed significant overall sequence similarity with other known proteins, HrpZ$_{Pss}$ contained a 24-amino acid sequence that is homologous with a region of the PopA1 elicitor protein of the tomato pathogen, *Pseudomonas solanacearum* GMI1000. *hrpA*, the upstream ORF, was highly divergent: The amino acid sequence identities of HrpA$_{Pss}$/HrpA$_{Psg}$ and HrpA$_{Pss}$/HrpA$_{Pst}$ were 91 and 28%, respectively, and a HrpA sequence showed similarity to known proteins. In contrast, the predicted products of the downstream ORFs in *P. s.* pv. *syringae* and *P. s.* pv. *tomato, hrpB, hrpC, hrpD,* and *hrpE* showed varying levels of similarity to those of *yscI, yscJ, yscK,* and *yscL*. These are colinearly arranged genes in the *virC* locus of *Yersinia* spp., which are involved in the secretion of the Yop virulence proteins via the type III pathway. The similarity of the Ysc proteins was generally stronger in comparisons with the *P. s.* pv. *tomato* Hrp proteins. The HrpZ proteins were purified by heat denaturation of contaminating proteins followed by ammonium sulfate fractionation, hydrophobic chromatography, and gel electrophoresis. All three HrpZ proteins elicited the HR in tomato, whereas none of them elicited significant necrosis in soybean. The results indicate that HrpZ is encoded in an operon containing some of the genes involved in its own secretion and suggest that HrpZ structure does not directly determine bacterial host range.

Phytopathogenic strains of *Pseudomonas syringae* cause two patterns of necrosis when the bacteria invade a plant. On a susceptible ("compatible") host, a necrotic lesion often develops over a period of days, with necrosis spreading as the bacteria multiply and the plant becomes diseased. On a resistant or nonhost plant, a localized cellular necrosis is induced within 24 to 48 h, and bacterial multiplication is inhibited. This was first reported by Klement (1963; Klement et al. 1964), who observed that when high concentrations of pathogenic bacteria are infiltrated into an incompatible plant they elicit a visible necrosis which is limited to the infiltrated area. This reaction, called the hypersensitive response (HR), involves localized cell death and production of anti-microbial compounds at the site of pathogen invasion (Bonas 1994). The ability of *P. syringae* and other nontumorigenic, gram-negative, bacterial pathogens to elicit the HR is governed by *hrp* genes. Typical Hrp⁻ mutants are pleiotropically defective in planta: They do not elicit the HR in nonhosts and they fail to multiply and cause disease in host plants (Lindgren et al. 1986). Clusters of *hrp* genes have been identified in many gram-negative phytopathogenic bacteria (Bonas 1994). A 25-kb *hrp* cluster from *P. s.* pv. *syringae* 61 is sufficient to confer the tobacco HR phenotype, but not the pathogenic phenotype on nonpathogenic bacteria (Huang et al. 1988). *hrp* genes have also been cloned and characterized extensively from *P. s.* pv. *phaseolicola* NPS3121, *P. solanacearum* GMI1000, *Xanthomonas campestris* pv. *vesicatoria* 75-3, and *Erwinia amylovora* Ea321 (Lindgren et al. 1986; Boucher et al. 1987; Beer et al. 1991; Bonas et al. 1991). Certain *hrp* genes are widely conserved among these pathogens, and several encode components of a protein secretion pathway that is similar to the type III pathway used by *Yersinia, Shigella,* and *Salmonella* spp. to secrete extracellular proteins involved in animal pathogenesis (Van Gijsegem et al. 1993). One activity of the *hrp*-encoded secretion pathway in phytopathogenic bacteria is the secretion of proteinaceous elicitors of the HR, which are also encoded by *hrp* genes.

The first *hrp*-encoded elicitor characterized was harpin from *E. amylovora* (Wei et al. 1992). Similar elicitors have since been isolated from other bacteria, including *P. s.* pv. *syringae* 61, *P. solanacearum* GMI1000, and *E. chrysanthemi*

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EC16 (He et al. 1993; Arlat et al. 1994; Bauer et al. 1994). Proteins in this family of elicitors share several general characteristics. They are glycine rich, heat-stable, lack cysteine, and appear highly susceptible to proteolysis. They lack an N-terminal signal peptide, but they are secreted to the bacterial milieu. Their expression and secretion is dependent on *hrp* genes. The biological role of these proteins in pathogenesis has not yet been determined, but the purified proteins can induce an HR on a nonhost plant such as tobacco. However, there are significant differences in the organization of the elicitor operons and the activity of the elicitors, which suggests that the *Erwinia* harpins, the *P. syringae hrpZ* product and the *P. solanacearum popA* product may represent three distinct classes of elicitors. In this work we will refer to the *P. s.* pv. *syringae* elicitor as HrpZ$_{Pss}$ rather than harpin$_{Pss}$. (He et al. 1993). This distinction is supported by the weak similarity of the amino acid sequences of the four proteins, with the only exception being the C-terminal halves of the *Erwinia* harpins (Bauer et al. 1994).

The location of known elicitor genes in reference to the *hrp* cluster varies in *P. s.* pv. *syringae*, *P. solanacearum*, and *E. amylovora*. *hrpN* and *hrpZ* are contiguous or within the *hrp* cluster, whereas *popA* lies outside (although near) the *P. solanacearum hrp* cluster (Wei et al. 1992; He et al. 1993; Arlat et al. 1994). There are no genes downstream of the elicitor gene in either the *hrpN* or the *popA* operons, which means that mutations in the elicitor genes do not have a polar effect on the Hrp phenotype, and mutant construction is straightforward. In contrast, mutagenesis and complementation studies of the *hrp* cluster from *P. s.* pv. *syringae* 61 have indicated that *hrpZ* lies upstream of at least one other *hrp* gene within an operon (Huang et al. 1991; Xiao et al. 1992).

In *E. amylovora* and *E. chrysanthemi*, harpins have been demonstrated to be sufficient and necessary to elicit the HR, and mutation of *hrpN* in *E. amylovora* has shown that harpin$_{Ea}$ is required for pathogenesis (Wei et al. 1992). However *hrpN* mutants of *E. chrysanthemi* can establish infections, albeit at a significantly reduced frequency, which suggests that harpin$_{Ech}$ is important but not essential for pathogenesis (Bauer et al. 1995). In contrast, a *popA* mutant of *P. solanacearum* is fully pathogenic on susceptible hosts, indicating that PopA1 is not required for pathogenesis (Arlat et al. 1994).

These elicitors may play a role in controlling the host specificity exhibited by *E. amylovora* and plant pathogenic pseudomonads such as *P. syringae* and *P. solanacearum*. However it is difficult to compare the activity of HrpZ$_{Pss}$ and harpin$_{Ea}$ in host and nonhost plants because legumes and rosaceous plants, the hosts of *P. s.* pv. *syringae* 61 and *E. amylovora* Ea321, respectively, respond poorly to preparations of any of these elicitor proteins (Wei et al. 1992; He et al. 1993). PopA1 from *P. solanacearum* does appear to act in a host-specific manner, inducing an HR on resistant lines of petunia and the nonhost tobacco, but not on susceptible lines of petunia or tomato (Arlat et al. 1994). This phenotype is similar to that of *avr* genes, but PopA1 is distinct from known Avr proteins in eliciting the HR directly on resistant plants. Harpin$_{Ech}$ elicits an HR on some compatible hosts of *E. chrysanthemi*, but in contrast to the other three bacteria *E. chrysanthemi* is a broad-host range pathogen and the activity of harpin$_{Ech}$ may not be representative of elicitor activity in a highly host-specific system (Bauer et al. 1995).

In previous work we cloned and characterized the *hrpZ* gene from *P. s.* pv. *syringae* 61, a weak pathogen of bean, and demonstrated with S uthern and immunoblots that other pathovars of *P. syringae* contain homologs of this gene (He et al. 1993). This supported the hypothesis that HrpZ represents a family of elicitors common to all pathogenic strains of *P. syringae*. We report here the isolation of homologs of HrpZ$_{Pss}$ from two other experimentally important pathovars of *P. syringae-P. s.* pv. *tomato* and *P. s.* pv. *glycinea*. Examining HrpZ from these three pathovars enabled us to look within this family of elicitors for variations in sequence and activity which could indicate a role in host range determination. In addition, we characterized the two genes flanking *hrpZ* in *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* and the entire *hrpZ* operon of *P. s.* pv. *tomato*. In conjunction with an accompanying paper (Huang et al. 1995), this completes the sequence of the *P. s.* pv. *syringae* 61 *hrp* genes carried on pHIR11 and provides clues to the function of the genes downstream of *hrpZ*. A preliminary account of portions of this work has been published (Collmer et al. 1994).

## RESULTS

**Cloning *hrpZ* from *P. s.* pv. *tomato* and *P. s.* pv. *glycinea*.**

We previously used Southern hybridization to demonstrate that both *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000 contain sequences homologous to a 0.75 kb *Bst*XI internal fragment of *hrpZ* from *P. s.* pv. *syringae* (He et al. 1993). The same probe was used to screen genomic libraries of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. The libraries were constructed in *E. coli* DH5α by inserting 8- to 12-kb fragments from partial *Sau*3AI digests of genomic DNA into the *Bam*HI site of pUCP19. The screen identified two plasmids with inserts of approximately 10 kb: pCPP2201 (*P. s.* pv. *tomato*) and pCPP2200 (*P. s.* pv. *glycinea*). The same *Bst*XI fragment was used to probe a Southern blot of pCPP2201 and pCPP2200 digested with *Bam*HI, *Eco*RI, and *Pst*I. The probe identified two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200 respectively (Fig. 1). The two *Pst*I fragments were cloned into the *Pst*I site of pBluescript II SK(−) (Stratagene, La Jolla, CA) in *E. coli* DH5α to create the plasmids pCPP2202 to pCPP2205, with the inserts in both orientations with respect to the *lac* promoter. Cell lysates of *E. coli* DH5α containing pCPP2203 (*hrpZ$_{Pss}$* in the vector promoter orientation) and pCPP2202 (*hrpZ$_{Pss}$* in the vector promoter orientation) induced an HR on tobacco, but those from cells containing pCPP2205 (*hrpZ$_{Pss}$* in the opposite orientation of the vector promoter) and pCPP2204 (*hrpZ$_{Pss}$* in the opposite orientation of the vector promoter) did not. HR activity was retained after incubating the lysate for 10 min at 100°C and removing denatured proteins by centrifugation. Insensitivity to heat treatment is a characteristic feature of previously isolated HR elicitors. Proteins in the lysates were separated on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies raised against purified HrpZ$_{Pss}$. Cross-reacting proteins of a similar size to HrpZ$_{Pss}$ were observed and provisionally named HrpZ$_{Pss}$ and HrpZ$_{Pst}$ (Fig. 2, lanes 2 and 4).

The intensity of the HrpZ$_{Pss}$ and HrpZ$_{Pst}$ bands was quite low in comparison to the band for HrpZ$_{Pss}$ expressed from pSYH10 in *E. coli* DH5α (Fig. 2, lane 1). This implied either

that expression was low due to the distance of the cloned gene from the *lac* promoter or that $HrpZ_{Psg}$ and $HrpZ_{Pst}$ did not hybridize strongly to the antibodies. A band corresponding to $HrpZ_{Pss}$ from pSYH10 could be clearly seen on a Coomassie-stained gel, but the bands for $HrpZ_{Psg}$ and $HrpZ_{Pst}$ were indistinct, which implies that low expression was a primary reason for the low signal. In an attempt to improve the level of expression of $HrpZ_{Psg}$ and $HrpZ_{Pst}$ we subcloned *Eco*RI-*Bam*HI fragments containing the inserts from pCPP2202 and pCPP2203 behind the T7 promoter of pET21(+) in *E. coli* BL21(DE3) to create the plasmids pCPP2206 and pCPP2207.

The T7 promoter enabled a moderate improvement in protein expression (Fig. 2, lanes 3 and 5).

A common arrangement of ORFs in the *hrpZ* operons of *P. s.* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* revealed by DNA sequence analysis.

Previously, we determined the complete nucleotide sequence of *hrpZ* from *P. s.* pv. *syringae* by sequencing a 1.4-kb subclone of pHIR11 (a cosmid containing the entire *hrp* cluster from *P. s.* pv. *syringae*) (He et al. 1993). In addition, analysis of the complementation groups and transcriptional
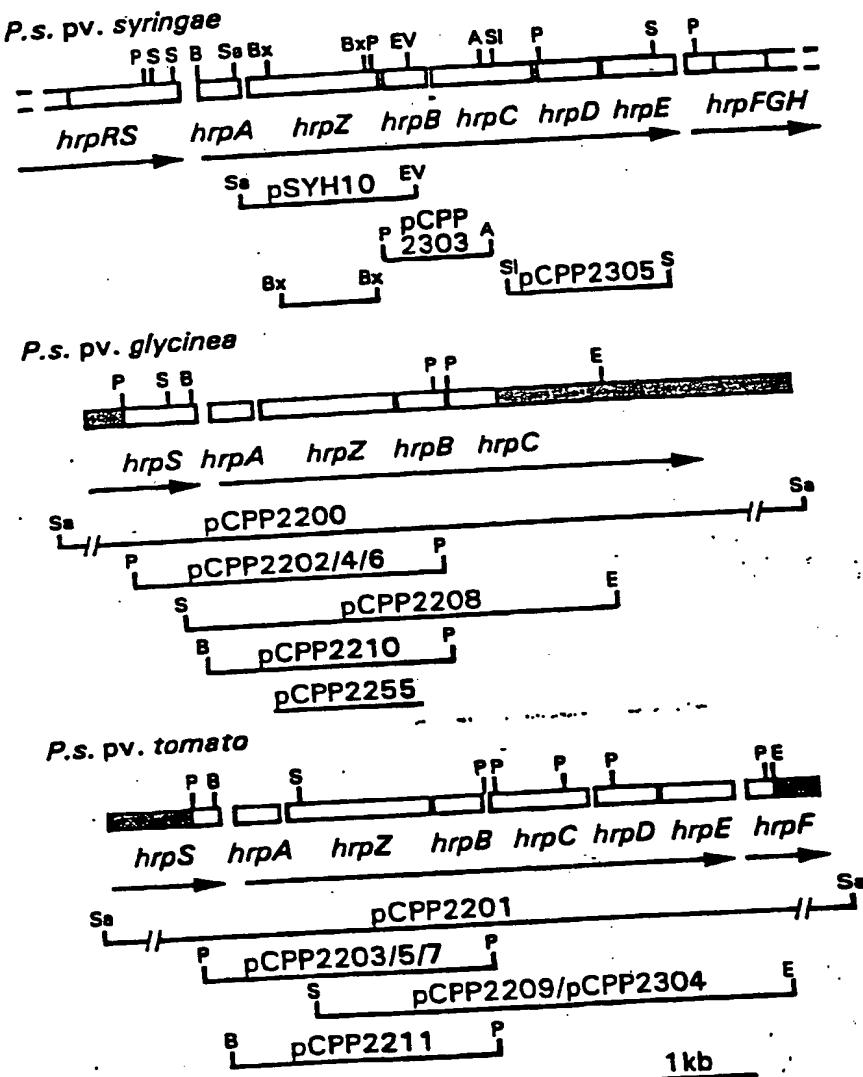


Fig. 1. Physical maps of the *hrpZ* regions from *Pseudomonas syringae* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4, and *P. s.* pv. *tomato* DC3000 and clones used in this study. Open boxes represent sequenced ORFs; filled boxes represent unsequenced DNA. Direction of transcription is indicated by the arrows. The 0.75-kb *Bst*XI fragment from *hrpZ_{Pss}* used as a probe for *hrpZ* genes in other pathovars is also shown. Key restriction sites within the sequenced regions are indicated, along with the subclones used in this study. Restriction endonuclease abbreviations: A, *Age*I*; B, *Bgl*II; Bx, *Bst*XI*; E, *Eco*RI; EV, *Eco*RV*; P, *Pst*I; S, *Sac*I; Sa, *Sau*3A*; SI, *Sal*I*. * Not all sites are shown.

units of pHIR11 using Tn*phoA* and Tn5-*gus*A1 mutagenesis (Huang et al. 1991; Xiao et al. 1992) suggested that *hrpZ* lay within an operon, upstream of at least one other *hrp* gene. Further subclones f pHIR11 were used to determine the sequence of the entire *hrpZ*ₚₛₛ peron (this study, Huang et al. 1995). We also determined the sequence of (i) the 2.2- and 2.4-kb *Pst*I subclones from pCPP2201 (*hrpZ*ₚₛ') and pCPP2200 (*hrpZ*ₚₛ'), (ii) an overlapping 3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 (designated pCPP2209), and (iii) part of an overlapping 3.6-kb subclone from pCPP2200 (designated pCPP2208), as shown in Figure 1. This yielded the sequence of the entire *P. s.* pv. *tomato hrpZ* operon and the first half of the *P. s.* pv. *glycinea* operon. The sequenced region of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* extends from *hrpS* (Xiao et al. 1994), through the *hrpZ* operon to the beginning of the *hrpH* operon (Huang et al. 1992), demonstrating that the organization of this region of the *hrp* cluster is conserved in both pathovars.

Codon preference analysis of the DNA sequence, using *P. s.* pv. *syringae* codon usage data, predicted that *hrpZ* was the second of six ORFs, all oriented in the same direction, an arrangement conserved in *P. s.* pv. *tomato* and at least the first four ORFs of *P. s.* pv. *glycinea*. The sequence of the noncoding DNA is shown in Figure 3. Five of the six ORFs have clear potential ribosome binding sites. The fifth ORF has a putative ribosome binding site in *P. s.* pv. *syringae*, but the site in *P. s.* pv. *tomato* is less clear, the initiation codon shown being selected by alignment with the ORF in *P. s.* pv. *syringae*. In the absence of recognizable terminator elements downstream of the first five ORFs it seems likely that the six ORFs represent a single operon, transcribed from upstream of the first ORF. The five predicted ORFs were provisionally named *hrpA* through *hrpE*, as shown in Figures 1 and 3.



kD  1  2  3  4  5  6  7

41.8 —

30.6 —

17.8 —
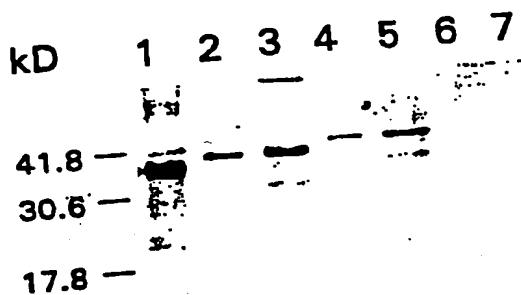
Fig. 2. Immunoblot showing expression of cloned *hrpZ* in *E. coli*. Cultures were grown in LM to an OD₆₀₀ of 0.8 to 1.0 at 30°C, collected by centrifugation and resuspended in 5 mM MES, pH 5.5. For lanes 3, 5 and 7, and 4, T7 expression was induced with 1 mM IPTG when the cells reached an OD₆₀₀ of 0.6, 3 h prior to collection. The cells were disrupted by sonication, and the crude lysate was partially purified by removal of the insoluble fraction after incubating the samples at 100°C for 10 min. SDS-loading buffer was added and the samples were incubated at 100°C for 2 min. The proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), probed with anti-HrpZₚₛₛ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α (pSYH10) (HrpZₚₛₛ); 2, *E. coli* DH5α (pCPP2202)(HrpZₚₛ); 3, *E. coli* BL21(DE3) (pCPP2206)(HrpZₚₛ); 4, *E. coli* DH5α (pCPP2203)(HrpZₚₜ); 5, *E. coli* BL21(DE3) (pCPP2207)(HrpZₚₜ); 6, *E. coli* DH5α (pBluescript II); 7, *E. coli* BL21(DE3)(pET21+).

A *hrp/avr* promoter consensus sequence lies upstream of the *hrpZ* operons of the three *P. syringae* pathovars.

The conserved sequence GGAACC—16bp—CCACNNA lies 50 bp upstream of the initiation codon of *hrpA* in all three pathovars (Fig. 3). This motif has been identified in the promoter regions of many *avr* and *hrp* genes (Innes et al. 1993; Shen and Keen 1993), and appears to be involved in positive regulation by HrpL, a putative alternative sigma factor which is itself positively regulated by HrpR and HrpS (Xiao and Hutcheson 1994). HrpL is a member of a family of alternative sigma factors, many of which are involved in secretion of extracellular factors in response to environmental stimuli (Lonetto et al. 1992). The presence of this promoter motif further supports the suggestion that the six ORFs form a single transcriptional unit which is regulated in a *hrp*-dependent manner. This motif can also be found beyond *hrpE*, upstream of *hrpFGH* in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, as indicated at the bottom of Figure 3, suggesting that the latter three ORFs form an independent *hrp*-regulated transcriptional unit in these two pathovars.

## Comparison of the HrpZ proteins of the three *P. syringae* pathovars.

The predicted amino acid sequences for HrpZ from each of the three pathovars are aligned in Figure 4. Although the proteins migrate slightly anomalously on an SDS polyacrylamide gel, the relative sizes of the estimated molecular weights correspond to the predicted values, with HrpZₚₛₛ being the largest of the three proteins (36.5 kDa), followed by HrpZₚₛ (35.3 kDa) and HrpZₚₜ (34.7 kDa). Amino-terminal sequencing of the first 10 to 15 residues of purified HrpZₚₛₛ and HrpZₚₜ confirmed the predicted initiation codons of both proteins, which aligned with the start codon of HrpZₚₛ as shown in Figures 3 and 4. The proteins expressed in *E. coli* appear to be the same size as those recovered from the supernatants of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*, indicating that the cloned gene is intact and that there are no large posttranslational modifications or deletions of HrpZ taking place in *P. syringae* but not in *E. coli*.

The amino acid sequence of HrpZₚₛₛ is quite highly conserved with respect to HrpZₚₛ, having 87% similarity and 79% identity. HrpZₚₜ is less conserved with respect to the two other proteins, with 75% similarity and 63% identity to HrpZₚₛₛ and HrpZₚₛ. However, the physical features of HrpZₚₜ and HrpZₚₛ are almost identical to those reported for HrpZₚₛ (He et al. 1993). All three are glycine-rich proteins lacking cysteine and tyrosine. HrpZₚₜ is the most glycine rich, being 15.7% glycine. The proteins lack the hydrophobic signal sequence used to target proteins for secretion via the Sec export pathway (Pugsley 1989). Analysis of the amino acid sequence fails to identify any obviously significant secondary structure, which is consistent with their sensitivity to proteases, and supports the suggestion that they adopt a fairly open structure in aqueous solution.

In our previous analysis of HrpZₚₛₛ (He et al. 1993), we noted the presence of two sets of short, direct repeats. Only one of these repeats, GGGLGTP, is conserved in the three proteins, with the substitution of a serine for threonine in the first repeat of both HrpZₚₛₛ and HrpZₚₛ. The significance f these repeats, if any, is unknown. A database search with each of the three proteins using the BLAST algorithm (Altschul et

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syringae    TTTTTTGCAG AAGATCTGGA ACCGATTCGC GGACACATGC CACCTAGCTG
glycinea    TTTTTTGCA. GAGCGCTGGA ACCGATTTAA GGGTCGTTAC CACTA.TCTG
tomato      TTTTTTGCAA AGACGCTGGA ACCGTATCGC AGGCTGCTGC CACTAGTGAG

syringae    TACCAAGCAA TTACGCTGGT ACAGACGAAG GGGTATGACG TTATG-----
glycinea    TACCAAGCAA TTACGCTGGT ACAGACCAAG GGGTATCACG TTATG-----
tomato      TACCAAGCAA TCACGCTGGT AAATCTTAAG GGGCATCAAA TCATG-----

syringae    ---------- --321bp--- ----------TGATTTCTTG. ACGCCCCTTC
glycinea    ---hrpA---  --321bp--- ----------TGATTTCTTGA ATGCCCCCAT
tomato      ---------- --336bp--- ----------TAATTATTTCT GATTGCCCCC

syringae    ATACCTGAGG GGGCTGCTAC TTTTAGGAGG TTGTG..ATG ----------
glycinea    CACACAGAGG GGGCTGCTAC TTTGAGGAGG TTGTG..ATG ----------
tomato      TCATCAGAGG GGGCCGCTAC CTTGGGATGG GCGTTTTATG ----------
            =>      <===  ====

syringae    ---------- --1020bp-- -.........-- ---------- ----------
glycinea    ---hrpZ--- --1032bp-- -.........-- ---------- ----------
tomato      ---------- --1107bp-- -.--------- ---------- ----------

syringae    ---------- ---------- ---------- ---------- ----------
glycinea    ---------- ---------- ---------- ---------- ----------
tomato      ---------- ---------- ---------- ---------- ----------

syringae    TGACCGACAA CCGCCTGACG GAGAACTCAC GTG------- ---hrpB---
glycinea    TGACTGATAC CCGCCTGACG GAGAACTCAC GTG------- ---hrpB---
tomato      TCACTGACAG CCGCCTGACG GAGAACCAGT GTG------- ----------

syringae    --369bp--- ---------- ----------TAGAGGTTTC CGTG------ ----------
glycinea    --369bp--- ---------- ----------TAGAGGTTCT CGTG------ ----------
tomato      --369bp--- ---------- ----------TAGAGGTTTC CGTG------ ----------

syringae    ---------- --801bp--- ---------- ---------- ----------
glycinea    ---hrpC--- incomplete ---------- ---------- ----------
tomato      ---------- --801bp--- ---------- ---------- ----------

syringae    ---------- -----TCATG GACCTGACCG CCGAGGACTA TTGGACTCAG
tomato      ---------- ----ATGATG AGCCTTTCTG CCGAGGATCA CTGGATTCAC

syringae    TGGTGGTGCA ATCCCTGGCC ATGGGCGCAT CCGGGCTGGC AAAGCCGGTT
tomato      TGGTGGTGCA ACCCCTGGCA GTGGGCACAT TCGGAGTGGC ATGACCGATT

syringae    CGCCGAGCGC TGCGGACTGA CCGTCAGCGA ATGTGAAGCC CTTATG----
tomato      CGCCAACGCT CGTGGGTTAT CGGTCAGTGA CTGCGATGCG CTCATG----

syringae    ---------- ---hrpD--- --396bp--- ---------- ----------
tomato      ---------- ---------- --396bp--- ---------- ----------

syringae    --TGAGTAT. .CCGCTCCTC TCTGCACCAG GAATTCTCCC ATG-------
tomato      --TGAATCCG AACCAGCTTC TCTGCATCAG GAATACGCCC ATG-------

syringae    ---hrpE--- --576bp--- ---------- ---------- --------TGA
tomato      ---------- --576bp--- ---------- ---------- --------ATG

syringae    AACAGACT.. .........C TTGCGGCGAA AATGGAACCG CTCCACCTGT
tomato      TACACACTCT CTGCACTCAC TTGATCGCAT GATGGAACCG CTCGGCGGGT

syringae    TTGCTCCACT CAAGGTTTGA ACCTTTCTGC TGGAGTATCA GGACATG
tomato      TTGCTCCACT CAAGGTTTGA ACCCTTCTGC TGGAGCACCA GGACATG
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Fig. 3. Nucleotide sequences of the noncoding regions of the *hrpZ* operon from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*. The sequences flanking the six ORFs of the *hrpZ* operon were aligned using the PILEUP algorithm (Genetics Computer Group). For *P. s.* pv. *syringae* and *P. s.* pv. *tomato* the sequence extends from immediately downstream of *hrpS* to the end of the operon. For *P. s.* pv. *glycinea* the sequenced region terminates at the beginning of *hrpC*. The proposed initiation and termination codons are highlighted for each ORF. The *hrp/avr* consensus sequences upstream of *hrpA* amd *hrpF* are marked by double lines, with the conserved nucleotides in bold and the putative ribosome binding sites for each ORF underlined. A short inverted repeat upstream of *hrpZ* is also indicated with dashed arrows.

al. 1990) did n t find significant homology to any other bacterial proteins, with the exception of a single, glycine rich region f und only in HrpZ$_{Ps}$ (Fig. 4). This stretch of 24 amino acids has homology at both the nucle tide and amino acid level to a region of the host-specific elicitor PopAl from P.

*solanacearum*, as shown at the bottom of Figure 4. There is no overall similarity of the amino acid and nucleotide sequences of HrpZ to the HR elicitors characterized from *E. amylovora*, *E. chrysanthemi*, and *P. solanacearum* xcept to a degree accounted for by their similar composition.



Fig. 4. Alignment of the protein sequences of HrpA and HrpZ. The predicted protein sequences of HrpA and HrpZ from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* were aligned using the PILEUP algorithm (Genetics Computer Group). The alignment of a unique glycine rich region of HrpZ$_{Ps}$ with a homologous region of PopAl from *P. solanacearum* is also shown.

The predicted HrpA protein of *P. s.* pv. *tomato* differs substantially from that of *P. s.* pv. *syringae* and *P. s.* pv. *glycinea.*

The first ORF of the *hrpZ* operon starts 50 bp downstream of th conserved *hrp/avr* promoter motif, as shown in Figure 3. The predicted product is a small (11 kDa), hydrophilic protein with a hydrophobic N-terminus. An alignment of the amino acid sequences from all three pathovars is shown in Figure 4. Although the predicted sequences of HrpA from *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* are highly conserved, with 92% similarity and 91% identity to each other, HrpA from *P. s.* pv. *tomato* is quite divergent, having only 42% similarity and 28% identity to HrpA from *P. s.* pv. *syringae* The presence of a ribosome binding site and the highly conserved character of HrpA in two of the three pathovars supports the hypothesis that HrpA is translated. T7 polymerase-dependent expression of *hrpA* (described below) provides further evidence for production of a HrpA protein. Cell lysates of *E. coli* expressing only HrpA did not elicit the HR n t bacco (data not shown), which suggests that it does not contribute directly to the HR. The role of HrpA in the bacterium is unknown, and it shows no significant homology to any previously characterized proteins.

**T7 expression studies.**

To confirm the production of proteins corresponding to the two sets of newly cloned *hrpA* and *hrpZ* genes, the *BglII-PstI* fragments from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were subcloned into pET21(+) and the products specifically labelled by T7 promoter/polymerase-dependent expression in *E. coli* BL21(DE3) cells incubated with [$^{35}$S]-methionine (Studier et al. 1990). Radiolabeled proteins in the cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Lysates of cells containing pCPP2211 displayed unique bands which corresponded well with the predicted molecular weight of HrpA (11.5 kDa) and were consistent with the previously observed mobility of HrpZ$_{Pst}$ (Fig. 5, lane 2). Lysates of cells containing pCPP2210 contained bands corresponding to HrpZ$_{Psg}$ (36 kDa) and HrpA (11 kDa)(Fig. 5, lane 3). No HrpB band was visible in the products of pCPP2211 (Fig. 5, lane 2), but this could potentially be attributed to the omission of cysteine, which is not required for HrpA and HrpZ synthesis, from the amino-acids added to the reaction mixture. T7 expression of HrpB was independently confirmed for both *P. s.* pv. *syringae* and *P. s.* pv. *tomato* using a 0.84-kb *PstI-AgeI* fragment of pHIR11 and the 3.7-kb *SacI-EcoRI* fragment from pCPP2209, subcloned into LITMUS 28 to construct the plasmids pCPP2303 and pCPP2304. T7 expression in *E. coli* BL21(DE3) cells was performed as outlined above and in Figure 5. In each case a protein of about 13 kDa was observed, which corresponds well with the predicted molecular weight of HrpB from each of the two pathovars (data not shown). In an accompanying study Huang et al. (1995) have confirmed the production of proteins corresponding to HrpC, HrpD, and HrpE from *P. s.* pv. *syringae* 61. The similarities between the three pathovars suggest that the equivalent ORFs in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* also encode proteins. However when we independently confirmed the production of HrpD from *P. s.* pv. *syringae* 61 using a 1.3-kb *SalI-SacI* subclone from pHIR11 cloned into pT7-6 (pCPP2305) our results suggested the use

f an alternative initiation codon to make a larger (21 kDa) HrpD protein (data not shown). In the absence of a strong ribosome binding site at either of the putative initiation codons, the exact size of HrpD remains uncertain.

**The four ORFs d wnstream of *hrpZ* show varying similarities to *Yersinia* Ysc proteins.**

The *hrpC, hrpD,* and *hrpE* genes downstream of *hrpZ* in *P. s.* pv. *syringae* 61 have been sequenced and the products identified using T7 polymerase-dependent expression (Huang et al. 1995). Two of the predicted proteins, HrpC and HrpE, were shown to be homologous to the proteins YscJ and YscL, respectively, which are encoded in the *virC* operon of *Yersinia enterocolitica* and are involved in the type III secretion pathway (Michiels et al. 1991). Homologs of YscJ have also been found in the *hrp* clusters of several other phytopathogenic bacteria, including *P. solanacearum* and *X. campestris* (Fenselau et al. 1992; Gough et al. 1992). Additional homologs are *Salmonella typhimurium* FliF and *Rhizobium fredii* NoIT (Jones et al. 1989; Meinhardt et al. 1993). The same four downstream ORFs are found in *P. s.* pv. *tomato,* and the partial sequence of the operon from *P. s.* pv. *glycinea* confirms the presence of the first two of these ORFs, *hrpB* and *hrpC,* in this pathovar (Fig. 6).

HrpB is fairly conserved in all three pathovars, as shown by the alignment presented in Figure 6. It encodes a small serine-rich protein of approximately 13 kDa. BLAST searches using HrpB from either *P. s.* pv. *syringae* or *P. s.* pv. *glycinea* identified no significant homologies, but a search using HrpB from *P. s.* pv. *tomato* identified similarity to the *Yersinia* protein, YscI. YscI is 115 amino acids long, thus slightly shorter than HrpB (127 amino acids). *yscI* lies immediately upstream of *yscJ* in the *virC* operon, which suggests that the downstream ORFs of the *hrpZ* operon might be colinear with a region of the *virC* operon.
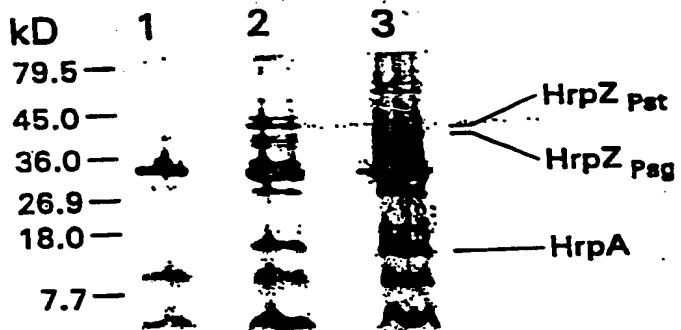


Fig. 5. T7 polymerase-dependent expression and radiolabeling of HrpA and HrpZ. T7 promoter/polymerase expression was carried out using the pET21(+) vector system in *E. coli* BL21(DE3). Cells were grown in LM to an OD$_{600}$ of 0.5, then centrifuged and resuspended in M9 minimal medium supplemented with 0.01% amino acids (lacking methionine and cysteine), glucose and thiamine. Cells were incubated at 30°C for 3 h and then induced with 1 mM IPTG for 10 min, followed by incubation with rifampicin at 300 µg/ml for 30 min. Cells were incubated with 10 µCi [$^{35}$S]-methionine for 10 min, lysed in SDS-loading buffer, and the proteins were separated by SDS-polyacrylamide electrophoresis and visualized by autoradiography. *E. coli* BL21(DE3) cells carried the following plasmids in lanes: 1, pET21(+); 2, pCPP2211; 3, pCPP2210.

The apparent colinear arrangement of this group of *hrp* and *ysc* genes led us to inspect the *P. s.* pv. *syringae* and *P. s.* pv. *tomato* HrpD proteins for possible similarity to the *Yersinia* spp. YscK proteins. The similarity between the HrpD of *P. s.* pv. *syringae* and *Y. pseudotuberculosis* was the highest, with 28% f the amino acids identical and 57% similar. The HrpD and YscK proteins are of similar overall composition, and they lack any predicted transmembrane segments. However, there is a striking discrepancy between the sizes of the two proteins. HrpD is only 133 amino-acids long, whereas YscK from *Y. pseudotuberculosis* is 209 amino-acids long. From the T7 experiments described above it is important to note that in the absence of a strong ribosome binding site, the precise ini-

tiati n codon of the *hrpD* ORF is uncertain; it is conceivable that *hrpD* actually initiates immediately downstream of *hrpC*, at the ATG codon which overlaps the stop cod n of *hrpC*, which would yield a predicted protein of 176 amino acids for HrpZ_Ps r 175 amino acids for HrpZ_Ps in an arrangement similar to that of the *yscJ* and *yscK* ORFs in *Yersinia* spp. However, this codon and all other potential initiation codons upstream of the one we have chosen lack ribosome binding sites, and the pattern of codon usage suggests that the intergenic region is not translated.

Although the similarities between HrpB/YscI, HrpD/YscK, and HrpE/YscL are lower than those involving HrpC/YscJ, the similarities of HrpB/YscI and HrpE/YscL are clearly in-



Fig. 6. Alignment of the protein sequences of HrpB from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*, and HrpC, HrpD and HrpE from *P. s.* pv. *syringae* and *P. s.* pv. *tomato* with YscI, YscJ, YscK, and YscL from *Y. enterocolitica* and *Y. pseudotuberculosis* (Michiels et al. 1991; Rimpilainen et al. 1992). *(continued on next page)*

dicative of probable hom logy as based on a difference between the scores for the optimized and the average f 100 rand m Gap alignments being at least 5 times the standard deviation for the randomized alignments (Doolittle 1986). The scores for HrpD/YscK lie at the margin of significance by this measure. However, the varying levels of similarity are consistent with the divergence observed between Hrp proteins from different *P. syringae* pathovars and between Ysc proteins from different *Yersinia* spp. The results for HrpB,C and E lend support to the weak homology of HrpD to YscK and suggest that *hrpB, hrpC, hrpD,* and *hrpE* are colinear with *yscI, yscJ, yscK,* and *yscL.*

In a recent report, Van Gijsegem et al. (1995) observe that the *P. solanacearum* GMI1000 *hrp* cluster also encodes homologs of YscJ and YscL but not YscI and YscK. It is possible that with relatively divergent Hrp sequences, similarities with Ysc proteins may be found only after examining the sequences from several plant pathogens. It is interesting to note that there is no ORF following *hrpE* that is homologous to the protein encoded by the final gene of the *virC* operon, YscM. However, the *hrpZ* operon lies immediately upstream of the *hrpH* operon (Fig. 1), and HrpH is a homolog of YscC, a secretion protein which lies upstream of *yscIJKL* within the *virC* operon (Michiels et al. 1991). This suggests that a sig-



Fig. 6. (continued from preceding page)

nificant proportion f the *virC* operon is conserved in *P. sy-ringae*, albeit in a rearranged form. Eckhardt (1978) gels of total DNA. Southern-bl tted and probed with a 0.75-kb *BstXI* internal fragment of *hrpZ*ₚₛₛ suggested that the *hrp* genes are chromos mal in the three strains of *P. syringae* studied, rather than being plasmid-borne as are the *hrp* genes of *P. solanacearum* GMI1000 or the *ysc* genes of *Yersinia* spp. (Van Gijsegem et al. 1993; data not shown). The homologies of the *hrpZ* operons are summarized in Table 1.

### Overexpression, purification, and biological assay of HrpZₚₛₛ and HrpZₚₜₒ

Partially purified lysates of *E. coli* expressing HrpZₚₛₛ and HrpZₚₜₒ elicited a clear HR on tobacco while control lysates f *E. coli* containing vector alone did not. However the activity of the cell lysates on the two host plants was more ambiguous. Soybean is generally unreactive to cell lysates from either pathogen, while tomato is quite sensitive and sometimes weakly reactive not only to cell lysates of *E. coli* expressing HrpZ, but also to control lysates of *E. coli* containing vector alone. To accurately evaluate the biological properties of HrpZ from each of the two pathovars, it was necessary to purify HrpZ. It was also necessary to ascertain that the HR observed on tobacco was due solely to HrpZ and not to the products of either of the two flanking ORFs, HrpA and HrpB, since HrpA and a fusion protein of HrpB were being expressed in addition to HrpZ by the original *hrpZ*ₚₛₛ and *hrpZ*ₚₜₒ clones.

As a first step towards purifying HrpZ, we attempted to increase the level of expression. From the sequence of the *PstI* clones encoding *hrpZ* it was clear that long stretches of DNA encoding *hrpA* and the 3′ end of *hrpS* (1,144 bp in *hrpZ*ₚₜₒ pCPP2202 and 809 bp in *hrpZ*ₚₛₛ pCPP2203) separated *hrpZ* from the *lac* promoter in pBluescript II. A series of deletions f the 5′ end of the *hrpZ*ₚₜₒ clone were constructed using the Erase-a-Base system (Promega), bringing the *lac* promoter within 100 bp of the *hrpZ* initiation codon, and removing *hrpA*. Although cell lysates expressing the deleted clones retained HR eliciting activity, they did not show a substantial increase in gene expression. Searching for an explanation for this behavior we identified a number of potential contributing

fact rs. The first possibility was the presence of a *cis*-acting sequence contained in the 100 bp remaining upstream f *hrpZ*ₚₜₒ. Using a terminator analysis program we identified a 9-bp inverted repeat located between *hrpA* and *hrpZ* (Fig. 3). Although this repeat lacks the AT-rich sequence downstream which is characteristic of many terminators, it is possible that its presence encourages premature transcription termination. Similar repeats, albeit with weaker secondary structure, can be found upstream of *hrpZ*ₚₛₛ and *hrpZ*ₚₜₒ. A second factor contributing specifically to the low expression of *hrpZ*ₚₛₛ may be the absence of a strong ribosome binding site. Finally, there could be factors related to the proteins themselves, such as a lack of stability.

To eliminate possible *cis*-acting sequences and to obtain clones of *hrpZ*ₚₛₛ and *hrpZ*ₚₜₒ that lack *hrpA* and *hrpB*, the *hrpZ* genes from both pathovars were amplified by PCR, directionally cloned into pBluescript II and transformed into *E. coli* DH5α F′*lacI*ᵠ. We obtained significantly increased expression of HrpZₚₜₒ using the plasmid pCPP2255 (Fig. 7), but unexpectedly, overexpression of HrpZₚₛₛ appeared to be deleterious to the cells, and plasmids recovered from transformants often showed rearrangements. To maximize expression of HrpZₚₛₛ under these conditions, we introduced subclones containing the gene behind the T7 promoter of pET21(+) (Novagen, Madison, WI). Unlike the *lac* promoter, the T7 promoter is less sensitive to distance effects, and expression of HrpZₚₜₒ in *E. coli* BL21(DE3), with pET21(+) as the vector, resulted in increased expression as shown in Figures 2 and 8. Expression in BL21(DE3) also allowed us to retain almost complete repression of *hrpZ* until induction with IPTG. Good expression of HrpZₚₛₛ was achieved using the plasmid pCPP2211 in *E. coli* BL21(DE3).

The quality of the samples obtained following partial purification of the lysates by heat treatment was quite variable. To ensure removal of the majority of the contaminating proteins and to obtain a more concentrated sample of protein, we further purified HrpZ by ammonium sulphate precipitation and hydrophobic chromatography, which as indicated in Figure 8, yielded a distinct band on a Coomassie-stained gel. Purified, active HrpZ could then be obtained by electroelution from excised gel slices. This procedure was also used to isolate

**Table 1.** Homologies of *Pseudomonas syringae* pv. *syringae hrpZ* operon proteins with proteins from other *P. syringae* pathovars and *Yersinia* spp.

| | HrpA (108)[a] | HrpZ (341) | HrpB (124) | HrpC (268) | HrpD (133)[d] | HrpE (193) |
|---|---|---|---|---|---|---|
| *P. s.* pv. *syringae* | (108) | (345) | (124) | (268) | (133) | (193) |
| *P. s.* pv. *glycinea* | 91/92[b] | 79/87 | 94/96 | 90/95 | 78/87 | 76/87 |
| *P. s.* pv. *tomato* | (108) 28/42 | (370) 63/75 | (124) 68/80 | YscJ (244) | YscK (203) | YscL (223) |
| *Y. enterocolitica* | | | Yscl (115) 22/45 | 35/59 38/60 | 26/53 22/48 | 21/47 22/46 |
| *Y. pseudotuberculosis* | | | 24/45 (115) 22/45 21/44 | (244) 35/59 38/60 | (209) 28/57 23/49 | (221) 21/47 22/46 |

[a] Number of amino acids in the protein is given in parentheses.
[b] Percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein; the second are in comparison with the *P. s.* pv. *syringae* protein; the second are in comparison with *P. s.* pv. *tomato*.
[c] The first pair of values are the percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein.
[d] The data presented here are for the shorter of the two potential ORFs encoding *hrpD*. The larger versions of the HrpD proteins of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* would be respectively 175 and 176 amino acids long with 74/84% identity/similarity to each other.

HrpZ from the supernatants of *P. s.* pv. *tomato* and *P. s.* pv *glycinea* grown in *hrp*-inducing minimal media (Fig. 9). Preparations of the purified HrpZ proteins from *P. s.* pvs. *syringae*, *glycinea*, and *tomato*, at a concentration of ≥20 μM in MES buffer, were infiltrated into the leaves of tobacco, soybean, and tomato. The three proteins elicited a collapse involving >50% of the infiltrated tissue in tobacco and tomato leaves that developed within 18 h and was typical of the HR elicited by incompatible *P. syringae* strains, but they caused no visible reaction in soybean. It is worth noting that tobacco and tomato plants vary substantially in their sensitivity to harpin preparations. For example, some leaves on sensitive tomato plants will respond to 2 to 5 μM HrpZ$_{Psn}$ but ≥20 μM is required for consistent results. Furthermore, unlike tobacco, tomato plants that have responded hypersensitively to a HrpZ preparation do not respond to subsequent infiltrations of the elicitor. The spurious necroses sometimes observed were deduced to result from mechanical damage incurred during infiltration or the infiltration of preparations contaminated with salts or containing high concentrations of vector control *E. coli* lysates. These necroses developed much more quickly (within 4 to 6 h), and were much weaker and patchier than the confluent HR elicited by HrpZ. The fact that the HR induced by HrpZ in tomato and tobacco is an active response of host tissue was confirmed by coinfiltration of either sodium vanadate at $5^{-3} \times 10^{-3}$ M or lanthanum chloride at $1 \times 10$ M. Each f these two inhibitors of plant metabolism completely inhibited the HR elicited by HrpZ preparations from each of the three pathovars but not the necrosis caused by the other factors mentioned.

## DISCUSSION

We have used the *P. s.* pv. *syringae* 61 *hrpZ* gene to isolate the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000. Characterization of the *hrpZ* genes, products, and flanking DNA of these three pathovars has revealed the structure of the *hrpZ* operon, the relative variation among

ORFs within the operon, the presence of g nes downstream of *hrpZ* that are colinear with a block of genes involved with *Yersinia* virulence protein secreti n, and the presence in HrpZ$_{Ps}$ of a sequence related to a sequence in the PopA1



Fig. 8. Overexpression and purification of HrpZ$_{Ps}$. Cultures were grown to an OD$_{600}$ of 0.6 and induced with 1 mM IPTG. HrpZ$_{Ps}$ was then partially purified from the cell lysate in a three-step process: first, by heat-treatment at 100°C as previously described, then by precipitation with ammonium sulphate at 30 to 45% saturation, and finally by binding to a hydrophobic resin (phenyl-sepharose) at 30% ammonium sulphate. A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, *E. coli* BL21(DE3)(pET21+); 2, *E. coli* BL21(DE3)(pCPP2211). B, Immunoblot of the samples shown in A, probed with anti-HrpZ$_{Ps}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.
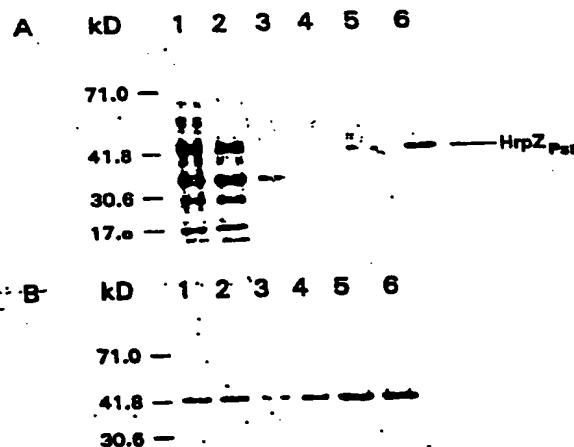




Fig. 9. Purification of HrpZ$_{Ps}$ from *hrp*-induced *Pseudomonas syringae* pv. *tomato*. Cells were grown in King's broth (KB) at 30°C and then resuspended in *hrp*-inducing minimal medium (Huynh et al. 1989) and incubated at room temperature overnight. Cells were removed by centrifugation and the supernatant heat-treated at 100°C for 10 min. Proteins in the supernatant were precipitated with ammonium sulphate at the percent saturations indicated. Proteins were desalted, concentrated, and resuspended in 5 mM MES using Centricon-10 tubes (Amicon). A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, supernatant extracted with Strataclean resin (Stratagene); 2, heat-treated supernatant extracted with Strataclean resin (Stratagene); 3, 0 to 20% ammonium sulphate fraction; 4, 20 to 30% ammonium sulphate; 5, 30 to 40% ammonium sulphate; 6, 30 to 45% ammonium sulphate. B, Immunoblot of the samples shown in A, probed with anti-HrpZ$_{Ps}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.



Fig. 7. Overexpression of HrpZ$_{Ps}$ in *E. coli* DH5α F'lacI$^q$. Cultures were grown overnight at 30°C in LM with 1 mM IPTG. Cell lysates were partially purified by heat treatment, separated on an SDS-polyacrylamide gel, transferred to Immobilon-P, immunoblotted with anti-HrpZ$_{Ps}$ antibodies, and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α F'lacI$^q$ (pCPP2255); 2 *E. coli* DH5α F'lacI$^q$ (pBluescript II).

protein of the tomato path gen *P. solanacearum* GM11000. We also observed that purified HrpZ$_{Ps}$ was-at least as effective as HrpZ$_{Pt}$ and HrpZ$_{Pg}$ in eliciting an HR-like necrosis in the leaves of tomato, a host of *P. s.* pv. *tomato* DC3000, whereas none of the HrpZ preparations elicited significant necrosis in soybean, the host of *P. s.* pv. *glycinea*.

**The HrpZ proteins of three *P. syringae* pathovars.**

A c mparison of the sequences of the three HrpZ proteins with each other and with HR elicitors characterized from other bacteria indicates that the HrpZ proteins represent a distinct family of elicitors that is conserved among *P. syringae* pathovars. The amino acid sequences of the three proteins are sufficiently similar to reveal their relatedness, but (with the exception of a sequence within HrpZ$_{Ps}$), they show no significant relatedness to elicitor proteins from other bacteria. Interestingly, *hrpZ* is the second most divergent ORF in the *hrpZ* operons of *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, with only 63% of the predicted amino acids being identical. Nevertheless, HrpZ$_{Ps}$, HrpZ$_{Pg}$, and HrpZ$_{Pt}$ are indistinguishable in several biological and physical properties. They have the same effect on different plants (discussed below), and they are heat stable, glycine-rich, and devoid of cysteine and tyrosine. The lack of tyrosine is a feature they differentially share with the *P. solanacearum* PopA1 protein but not the *Erwinia* harpins. This property has been speculated to allow the protein to avoid the H$_2$O$_2$-mediated cross-linking of tyrosine residues that may occur in plant cell walls during defense responses (Bradley et al. 1992; He et al. 1993).

Interestingly, a 24 amino acid, glycine-rich stretch of HrpZ$_{Ps}$ shows homology to part of PopA1, as does the cognate nucleotide sequence. The region of homology between HrpZ$_{Ps}$ and PopA1 corresponds exactly to the insertion in HrpZ$_{Ps}$. The insertion of this element within HrpZ$_{Ps}$ sequences that are otherwise similar among the three HrpZ proteins suggests horizontal transfer and a common ancestry with PopA1. Because the host range of *P. solanacearum* overlaps with that of *P. s.* pv. *tomato*, it is tempting to speculate that this region has some particular significance to pathogenesis on tomato, although, as discussed below, this is not obvious from the different effects of the two proteins on tomato.

The presence of this insert in active HrpZ$_{Ps}$ is another indicator of the apparent plasticity of structure/function relationships in these glycine-rich elicitor proteins. That significant changes to the structure of these proteins does not abolish their activity was previously demonstrated when a fortuitous *hrpZ$_{Ps}$* clone was found to produce an active derivative of HrpZ missing the N-terminal 125 amino acids, and the *popA* product was observed to be degraded in culture to an active form missing the N-terminal 93 amino acids (He et al. 1993; Arlat et al. 1994). Clearly the presence of this "additional" internal sequence does not diminish the ability of the protein to elicit the HR. In fact, although it is difficult to make a quantitative assessment, HrpZ$_{Ps}$ may actually be a slightly more potent elicitor of the HR than HrpZ$_{Pt}$.

However, HrpZ$_{Ps}$ appears to differ from the other HrpZ proteins in being deleterious to *E. coli* cells when overexpressed and is possibly more unstable, making it difficult to purify large amounts of the protein. Since the glycine-rich region is the most obvious difference between HrpZ$_{Ps}$ and HrpZ$_{Pt}$ it is possible that it contributes to this phenomenon.

We were able to overcome this problem experimentally by using a tightly regulated T7 promoter/polymerase system, but never obtained quite the same level of expression we achi ved with HrpZ$_{Ps}$ and HrpZ$_{Pt}$. However, there remains the obvious question of how HrpZ toxicity is avoided by *P. s.* pv. *tomato*. One possibility would be that HrpZ is never expressed at levels high enough to affect the bacterium, even when it is induced in planta. Some indirect evidence for this hypothesis is provided by our examination of the DNA upstream of *hrpZ$_{Ps}$*. The ORF has a weak ribosome binding site, and we also observed that expression of cloned *hrpZ* from the *lac* promoter appears to be attenuated by the presence of *cis*-acting upstream sequences. A 9-bp GC-rich repeat upstream of *hrpZ* may be significant in this regard. Preliminary data from northern blotting experiments also indicate that premature transcription termination may take place when *hrpA–hrpZ* clones are expressed in *E. coli* (G. Preston, unpublished). A second possibility is that the location of the *hrpZ* gene in an operon with secretion genes ensures tight coupling of synthesis and secretion. Genes encoding extracellular proteins and secretion pathway components are often coregulated, but with a few exceptions involving the type I pathway, they do not lie within the same operon (Fath and Kolter 1993). A third possibility is that *P. s.* pv. *tomato* is more tolerant of high levels of HrpZ than is *E. coli*, or it possesses a means of keeping HrpZ in a nontoxic form while it is in the cell.

Further comparison with the *Yersinia* virulence system presents an intriguing possibility in this regard. It has been shown that secretion of certain "Yops" (the *Yersinia* pathogenicity determinants), involves chaperone proteins, small hydrophilic proteins which help keep the Yop protein in a translocation competent form and help target it for secreti n (Wattiau et al. 1994). The genes encoding each chaperone are located adjacent to the gene encoding the corresponding Yop. Given the presence of several small ORFs of undetermined function in the pHIR11 *hrp* cluster, it is tempting to speculate that one of them, particularly *hrpA*, might encode a protein with chaperone function. There is a superficial resemblance between HrpA and *Yersinia* chaperones such as SycE. They are all small, hydrophilic, cytoplasmic proteins which lack a signal sequence, but there are no specific homologies. We are now constructing nonpolar mutations to test the role of HrpA in secretion. Preliminary results suggest that HrpA is not required for *E. coli* MC4100(pHIR11) to elicit an HR or secrete HrpZ (J. R. Alfano, unpublished), but in chaperone-mediated systems limited secretion of a protein will usually occur even in the absence of its chaperone, so it may be necessary to look quantitatively at secretion and accumulation of HrpZ to assess whether mutations in *hrpA* or other *hrp* genes have an effect.

**The colinear relationship between several *hrp* and *ysc* genes.**

From the sequence of the *hrpZ* operon it is clear that the parallels with the *Yersinia* type III secretion pathway extend beyond homologies of individual genes. The four genes downstream of *hrpZ*, *hrpB–E*, appear to be arranged colinearly with the region of the *virC* secretion operon from *Yersinia* that encodes YscI–L. The *virC* operon is a large operon containing 13 genes, *yscA-yscM*, several of which have been demonstrated to have a role in Yop secretion (Michiels et al. 1991). Of the four *Yersinia* genes with putative ho-

mologs in the *hrpZ* operon, only *yscJ* and *yscL* are known to have a role in secretion. An accompanying paper shows that five m re *hrp* genes, d wnstream of the *hrpH* operon, are colinear with the *yscQ–U* genes in the *virB* operon of *Yersinia* (Huang et al. 1995).

It appears that a significant proportion of the type III secretion pathway described in *Yersinia* can be identified in *P. syringae*, and it seems likely that increasing parallels between the two systems will be found. In both systems the secreted proteins are involved with early events in the interaction with the host, and expression of secretion genes and virulence proteins is tightly coregulated. The secretion pathway seems to function in a similar way, as in both cases secreted proteins lack an N-terminal signal peptide and are not posttranslati nally processed.

**HrpZ and host specificity.**

The function of HrpZ in compatible interactions is unclear. A likely role is the release of nutrients to the apoplast. Atkinson and Baker (1987a, 1987b) have proposed that the alkalinization of the apoplast caused by Hrp⁺ bacteria (which occurs at a slower rate in compatible interactions) results in the leakage of sucrose and other nutrients to support bacterial growth. One of the key unanswered questions regarding the *P. syringae* HrpZ proteins is their role in host specificity. Compatible interactions leading to disease are distinguished by the absence of the HR. Host-differential elicitor activity would be ne way to reconcile the production of HR-eliciting proteins by *P. syringae* and the phenomenon of host-specific compatibility. The failure of the PopA1 protein to elicit the HR in tomato, a host of *P. solanacearum* GMI1000, supports this concept (Arlat et al. 1994). Similarly, the isolated *P. s.* pv. *syringae* 61 HrpZ protein fails to elicit the HR in bean, although the significance of this is diminished by the fact that bean leaves appear insensitive to any harpins (He et al. 1993). To further explore this question, we infiltrated all three HrpZ proteins into the leaves of the host plants for each of the pathovars. The host plants of *P. s.* pv. *syringae* 61, and *P. s.* pv. *glycinea*, bean and soybean, respectively, are uniformly unreactive to HrpZ from both compatible and incompatible pathogens; however, tomato leaves proved to be highly sensitive to all three HrpZ proteins. Thus, our data argue against the hypothesis that host-differential activity of HrpZ proteins controls the host specificity of *P. syringae* pathovars.

If isolated HrpZₚₜ elicits the HR in tomato, why does *P. s.* pv. *tomato* not elicit the HR during pathogenesis? One possibility is that the response of tomato to HrpZₚₜ is qualitatively different than the response to HrpZₚₛ and HrpZₚₜ despite manifestation of the same gross morphology. That is, the necrosis elicited by HrpZₚₜ is fundamentally different than the HR and does not involve associated defenses that stop the pathogen. We are now testing this possibility with probes for HR-specific transcripts. A second possibility is that HrpZₚₜ production is regulated in a host-specific manner. However, *hrpZ* is clearly part of the Hrp regulon: *hrpZ* expression is transcriptionally linked with genes encoding components of the secretion pathway, the *hrpZ* operons in all three of these *P. syringae* pathovars have virtually the same *hrp/avr* promoter sequence, and expression of the *hrpZ* operon is likely required for pathogenicity. The conserved promoter sequences suggest that the *hrpZ* operon is regulated in *P. s.* pv. *glycinea*

and *P. s.* pv. *tomato* by the same nutriti nal conditi ns and HrpR, HrpS, HrpL regulatory cascade described for *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* (Grimm and Panopoulos 1989; Rahme et al. 1992; Xiao et al. 1992; Xiao et al. 1994; Xiao and Hutcheson 1994; Grimm et al. 1995). Whether differential expression of the Hrp regulon controls host specificity awaits determination. A third possibility is that the *P. syringae* pathovars produce host-specific suppressors of defense responses. This is supported by the observation that compatible pathogens do not trigger defense responses in host plants that are elicited by nonpathogens (Jakobek et al. 1993).

It is important to note that our data do not eliminate the possibility that the three HrpZ proteins actually have differential activity in host plants when delivered by living bacteria and that the HR observed may be an abnormal response resulting from the presentation of a high concentration of HrpZ in an artificial manner. In that regard, it is interesting that legumes, which appear insensitive to isolated harpins, respond to Hrp recombinant *E. coli* cells that secrete the same proteins (He et al. 1993). Experiments in which the *hrpZ* genes of *P. syringae* pathovars are switched or altered in their patterns of deployment should test more definitively the role of HrpZ in determining host specificity.

In conclusion, we have characterized an operon containing two components of the Hrp⁺ system of *P. syringae*—a block of secretion-related genes that are conserved in eukaryotic pathogens in the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Yersinia*, *Shigella*, and *Salmonella* and a gene encoding an elicitor that is unique to plant pathogens. The elicitors found in the *P. syringae* pathovars are a subfamily of a larger class that appears to be characteristic of plant pathogens, and which we postulate to have a role in releasing nutrients for bacterial utilization. Our challenge now is to determine how the various components of the Hrp system have been adapted to serve plant parasitism in the face of plant defenses.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.**

Bacteria and plasmids used in this study are shown in Table 2. Pseudomonads were routinely grown in King's B broth (King et al. 1954) at 30°C, but for certain experiments the *hrp*-derepressing minimal medium of Huynh et al. (1989), adjusted to pH 5.5, was used. *E. coli* was grown in LM (Sambrook et al. 1989) or terrific broth (Tartof and Hobbs 1987). Plasmids were introduced into bacteria by transformation (Sambrook et al. 1989) or electroporation (Gene Pulser, Bio-Rad).

**Plant materials.**

The plants used in this study were tobacco (*Nicotiana tabacum* L. 'Xanthii'), tomato (*Lycopersicon esculentum* Mill. 'Moneymaker'), and soybean (*Glycine max* L. 'Harosoy'). Plants were grown in a greenhouse or growth chamber at 23° to 25°C with a photoperiod of 16 to 24 h. Infiltration of plant leaves with HrpZ preparations was performed with blunt syringes as described (Huang et al. 1988).

**DNA analysis and sequencing.**

All DNA manipulations, except where specified, followed standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). The *hrpZ* region of pHIR11 was subcloned into

pBluescript II (Huang et al. 1995). Two PstI fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively, were subcl ned into pBluescript II SK(–) in b th orientations. A series of overlapping nested deletions covering both strands was generated for each of th subclones using Erase-a-Base (Promega, Madison, WI). The deletions were sequenced from double-stranded templates using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH) and forward and reverse M13 primers. Sequencing was completed using specific primers synthesized by Integrated DNA Technologies (Coralville, IA). In addition, the 3.7 and 3.6 kb SacI-EcoRI fragments, which overlap the PstI subclones from pCPP2201 and pCPP2200, were also subcloned into pBluescript II SK(–) and sequenced using the ABI 373A DNA sequencer at the Cornell Biotechnology Program DNA sequencing facility and specific primers synthesized by IDT. Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux et al. 1984). Homology searches against major sequence databases were d ne with the BLAST program (Altschul et al. 1990).

**PCR amplification of hrpZ from P. s. pv. glycinea and P. s. pv. tomato.**

The hrpZ genes of P. s. pv. glycinea and P. s. pv. tomato were amplified by PCR from the plasmids pCPP2202 and pCPP2203, respectively. Reactions were perf rmed using the PCR Optimizer kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reactions were overlaid with mineral oil and incubated in a Hybaid Thermal Reactor (Hybaid, Teddington, U.K.) using these cycle parameters: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, followed by a final incubation of 7 min at 72°C. The primers used for $hrpZ_{Psg}$ were 5′-TACGGGATCCTTTGAGGAGGTTGTGATG-3′ and 5′-TACGCTGCAGTATC AGTCAGGCAGCAGC-3′, and those for $hrpZ_{Pst}$ were 5′-TACGGGATCCATGCAAGCACTTA ACAGC-3′ and 5′-GGAACTGCAGCAAGCTCCGGCGA-TACAC-3′. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were designed to introduce a BamHI and a PstI site at the 5′ and 3′ ends, respectively, of each amplified fragment.

The $hrpZ_{Psg}$ fragment from pCPP2202 was successfully amplified in all reaction buffers tested. The $hrpZ_{Pst}$ fragment from pCPP2203 was successfully amplified using reaction buffer B (reaction concentration 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, pH 8.5). PCR products of the expected sizes of 1.0 and 1.2 kb were purified from an agarose gel, digested with PstI and BamHI, cloned into pBluescript II, and then transformed into E. coli DH5α F′lacI, yielding plasmid pCPP2255 carrying $hrpZ_{Pst}$. Plasmids containing

**Table 2. Bacterial strains and plasmids used in this study**

| Designation | Relevant characteristics[a] | Reference or source |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 Nal[r] | Hanahan 1983; Life Technologies, Inc. Grand Island, NY |
| DH5α F′lacI[q] | F′ proAB⁺ lacI[q]ZΔM15 zzf::Tn5[Km[r]]/φ80d lacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17 (r₁⁻m₁⁺) deoR thi-1 supE44λ⁻ gyrA96 relA1 | Life Technologies Inc. |
| BL21(DE3) | F⁻ ompT hsdB₈ (r₈⁻m₈⁻) dcm gal DE3 | Baker et al. 1987 |
| *Pseudomonas syringae* | | C. J. Baker |
| pv. *syringae* 61 | Wild type | D. E. Cuppels |
| pv. *glycinea* race 4 | Wild type | |
| pv. *tomato* DC3000 | Wild type, Rp[r] | Stratagene |
| Plasmids | | Schweizer 1991 |
| pBluescript II SK(-) | Cloning vector, Amp[r] | Novagen |
| pUCP19 | pUC19 derivative, Amp[r] | Tabor and Richardson 1988 |
| pET21(+) | T7 transcription vector, Amp[r] | New England Biolabs |
| pT7-6 | T7 transcription vector, Amp[r] | Huang et al. 1988 |
| LITMUS 28 | Cloning vector, Amp[r] | He et al. 1993 |
| pHIR11 | 25-kb cosmid containing P.s. pv. *syringae* 61 hrp cluster | This study |
| pSYH10 | $hrpZ_{Pss}$ ORF in pBluescript II | This study |
| pCPP2303 | 0.8-kb PstI-AgeI subclone from pHIR11, containing hrpB, in LITMUS 28 | This study |
| pCPP2305 | 1.3-kb SalI-SacI subclone from pHIR11, containing hrpD, in pT7-6 | This study |
| pCPP2200 | pUCP19 carrying 10-kb partial Sau3A1 fragment of P. s. pv. *glycinea* DNA with $hrpZ_{Psg}$ | |
| pCPP2202 | 2.4-kb PstI subclone of pCPP2200 in pBluescript II; $hrpA_{Psg}$ and $hrpZ_{Psg}$ in expressed orientation with respect to $P_{lac}$ | This study |
| pCPP2204 | As pCPP2202 but with $hrpZ_{Psg}$ in reversed orientation to $P_{lac}$ | This study |
| pCPP2206 | 2.4-kb PstI $hrpA_{Psg}$ and $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+) | This study |
| pCPP2208 | 3.6-kb SacI-EcoRI $hrpZ_{Psg}$ subclone from pCPP2200 in pBluescript II | This study |
| pCPP2210 | 1.85-kb BgIII-PstI $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+) | This study |
| pCPP2255 | PCR-amplified $hrpZ_{Pst}$ ORF in pBluescript II | This study |
| pCPP2201 | pUCP19 carrying 10-kb fragment of P. s. pv. *tomato* DNA with $hrpZ_{Pst}$ | This study |
| pCPP2203 | 2.2-kb PstI subclone of pCPP2201 in pBluescript II; $hrpA_{Pst}$ and $hrpZ_{Pst}$ in expressed orientation with respect to $P_{lac}$ | This study |
| pCPP2205 | As pCPP2203 but with $hrpZ_{Pst}$ in reversed orientation to $P_{lac}$ | This study |
| pCPP2207 | 2.2-kb $hrpZ_{Pst}$ subclone from pCPP2203 in pET21(+) | This study |
| pCPP2209 | 3.7-kb SacI-EcoRI subclone from pCPP2201 containing $hrpBCDE_{Pst}$ in pBluescript II | This study |
| pCPP2304 | 3.7-kb SacI-EcoRI subclone from pCPP2209 in LITMUS 28 | This study |
| pCPP2211 | 2.0-kb BgIII-PstI $hrpZ_{Pst}$ subclone from pCPP2203 in pET21(+) | This study |

[a] Amp[r] = ampicillin resistance; Nal[r] = nalidixic acid resistance; Rp[r] = rifampicin resistance.

PCR-amplified $hrpZ_{Ps}$ were found to be unstable and appeared to promote cell lysis.

## HrpZ purification and analysis.

HrpZ was purified from *E. coli* as previously described (He et al. 1993) with the following modifications. Cells were lysed in either 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5, or cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). For some experiments the supernatant from heat-treated lysate was partially purified after sonication by ammonium sulphate precipitation (25 to 45% saturation), with desalting and concentration being performed with Centricon-10 tubes (Amicon). For experiments requiring highly purified HrpZ expressed in *E. coli* BL21(DE3), the supernatant was further purified by binding to phenyl-sepharose (Sigma) in the presence of ammonium sulphate (>30% saturation) and elution with 5 mM MES, pH 5.5, f llowed by electrophoresis through a native 15% polyacrylamide gel. The purified protein was then eluted from excised gel slices using an Elutrap apparatus (Schleicher & Schuell) or from crushed gel slices using a Micropure separator (Amicon). Protein concentrations were determined using Bio-Rad protein assay solution. HrpZ was also purified from heat-treated supernatants of *P. syringae* grown in *hrp*-inducing medium (Huynh et al. 1989) by ammonium sulphate precipitation (25 to 45% saturation) and desalting/concentration using Centricon-10 tubes. For infiltration into plant tissue, HrpZ preparations were diluted to various degrees with 5mM MES, pH 5.5. The amino-terminal sequence analyses were performed at the Cornell Biotechnology Program Protein Analysis Facility ($HrpZ_{Ps}$) and the University of Kentucky Macromolecule Structure Analysis Facility ($HrpZ_{Ps}$).

## T7 expression and labeling of proteins in *E. coli*.

Proteins encoded by the *hrpZ* operon were expressed in *E. coli* BL21(DE3) by using the pET21(+) T7 expression system (N vagen). Conditions for isopropyl-β-D-thiogalactopyranoside (IPTG) induction of T7 RNA polymerase-dependent expression and labeling with L-[$^{35}$S]methionine were as described by Studier et al. (1990). After being labeled, cells were collected by centrifugation and then resuspended and lysed in SDS-loading buffer and the proteins re solved on an SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-ray film.

## Nucleotide sequence accession numbers.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers L41861 (*P. syringae* pv. *tomato hrpA, hrpZ, hrpB, hrpC, hrpD, hrpE*). L41862 (*P. syringae* pv. *glycinea hrpA, hrpZ, hrpB*), L41863 (*P. syringae* pv. *syringae hrpA*), and L41864 (*P. syringae* pv. *syringae hrpB*).

## ACKNOWLEDGMENTS

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